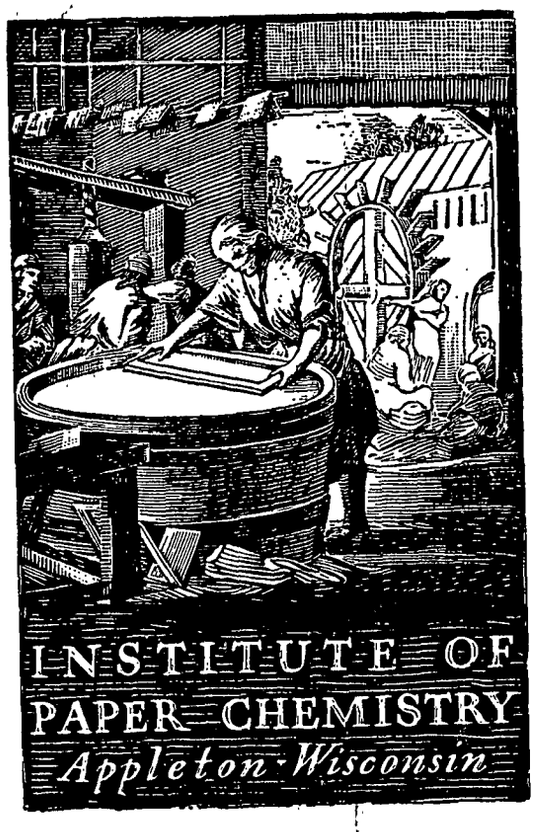

LABORATORY EVALUATION OF THE FUNGUS-INHIBITING PROPERTIES EXHIBITED BY THE VAPOR PHASE OF SIX EXPERIMENTAL MATERIALS

Project 1108-7-5

Progress Report One

to

FOURDRINIER KRAFT BOARD INSTITUTE, INC.

September 27, 1957

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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INTRODUCTION

At the present time biphenyl is the volatile fungistat used commercially to prevent the growth of molds on citrus fruit packaged in fiber containers. This material is both effective and economical; however, it would be unwise to consider biphenyl to be the most effective and economical material possible for such an application. This report presents the results of laboratory tests on six experimental compounds suggested as possible fungistats for use in citrus fruit packaging.

EXPERIMENTAL PROCEDURE

Two testing techniques were used in the study of these materials. The first was an agar-Petri dish method which has been used previously in the testing of volatile fungistats. The second method was developed to overcome several disadvantages noted during the first test.

In the test using the agar-Petri dish technique a small glass cup was centered in a Petri dish and 25 ml. of 1.5% malt extract agar (Trommer's Diastasic Extract of Malt) added to the dish surrounding the glass cup. The agar was allowed to solidify and the test material placed in the glass cup (0.1 g. or 1.0 ml. depending on whether the material was

a solid or a liquid). The agar was then inoculated with the test organisms and the Petri dish sealed with masking tape to prevent loss of the fungistatic vapors. Controls without fungistat and three replicate plates per variable were included in the test design. The plates were incubated at 28°C. and examined for mold growth at 3, 6, 10, 13, 19, 24, and 28 days.

As previously noted, the Petri dish method was not completely satisfactory in these tests. Five of the six test materials were liquids and the frequent handling of the plates during sealing or at the various inspection intervals occasionally spilled the liquid from the cups. The resulting contamination of the agar by the materials destroyed the "vapor inhibition" character of the test and made the results subject to question.

The second test was designed primarily to prevent contamination of the medium by the fungistatic materials. One-half pint fruit jars were used in place of Petri dishes as a sealable test chamber. The metal cover inserts were replaced with fiberboard discs made of heavy kraft cylinder board coated on one side with polyethylene film. The fiber inserts had several advantages over the metal inserts. The condensation that forms on the metal caps after steam sterilization of the jars caused considerable rusting during incubation. The condensate also dripped onto the surface of the agar and onto the test specimens. The fiber inserts absorbed what small condensate formed and eliminated this problem. The polyethylene film withstood autoclaving well and prevented vapor loss and drying out of the medium during incubation. A considerable saving in time resulted

from the use of fruit jars by eliminating the tedious masking tape operation.

Application of the fungistatic material was somewhat more involved than in the older method but introduced a meaningful dosage factor which did exist in the previous test. The treatment consisted of dipping squares of Whatman No. 1 filter paper into acetone solutions of the test materials. The squares were drained and the acetone allowed to evaporate. The pickup of fungistat by the paper squares was determined by weighing, although it was predicted with reasonably good results by an initial test with several solutions of varying concentration.

The size of the paper squares and the amount of fungistat per square were controlled to give a dosage equivalent to that found in commercial usage of biphenyl. Conversion of commercial rates of biphenyl application gives values of 1.95 mg./cm.^{2} on an area basis and 0.272 mg./cm.^{3} on a volume basis. The one-half pint jars have a volume of 310 cm.^{3}; therefore, the paper squares were made 1-13/16 inches on edge and treated with approximately 85 mg. per square with the various test materials to parallel commercial biphenyl usage.

The treated paper squares were suspended in the test jars on nichrome wire hooks attached to the fiber covers thus preventing any possible direct contamination of the medium with the test materials. The 25 ml. of 1.5% malt agar was added to the jars and inoculated with the test organisms in the same manner as the older method. Control jars contained paper squares

dipped in acetone only and two replicates were made of each test variable. The jars were incubated at 28°C. and examined after 3, 7, 10, 14, and 21 days.

The volatility of a material is of importance in commercial usage since a highly volatile material will dissipate rapidly and give protection for only a short period of time. Since it was a simple matter to treat a second series of paper squares in the second test this aspect of the test materials could also be examined. Ten replicate squares of each treatment were exposed to the air on wire racks and reweighed after varying intervals of time to determine the loss of material. Control squares dipped in acetone only were included to measure treatment effect and moisture changes due to humidity fluctuation.

The test materials with one exception were dialkyl derivatives of dichlorosuccinate. The dichlorosuccinates were obtained from the Allied Chemical and Dye Corporation. The other material (ASC-4) was sent to us by Stecker Chemicals, Incorporated, and the chemical composition is not known.

Test Materials

(1) Dimethyl dichlorosuccinate

(2) Diethyl dichlorosuccinate

(3) Di-n-propyl dichlorosuccinate

(4) Di-iso-propyl dichlorosuccinate

(5) Di-n-butyl dichlorosuccinate

(6) ASC-4

The compound ASC-4 was tested only by the older Petri dish method. The dichlorosuccinates will be referred to by their alkyl grouping in the report. Biphenyl was included in both tests for comparison purposes.

The test fungi used were Phomopsis citri and Diplodia natalensis which cause stem-end rot of citrus fruits, Penicillium italicum and Penicillium digitatum which cause blue and green molding of citrus fruits, and Rhizopus nigricans which causes soft rot of various fruits and vegetables. The inoculum was prepared by first growing the organism in 100 ml. of 1-1/2% malt broth for two weeks at 28°C. After incubation, the culture was diluted with 100 ml. of sterile water and disintegrated for about one minute in a Stevens Mixer. Then 10 ml. of the disintegrated culture was diluted to 100 ml. with sterile water, mixed well, and 1 ml. used to inoculate each test jar or Petri plate.

EXPERIMENTAL RESULTS AND DISCUSSION

The results of the Petri plate test are listed in Table I. It was apparent in this series that the Stecker material ASC-4 had very little if any inhibitory effect on the test fungi. The dichlorosuccinate derivatives, on the other hand, gave very good results--all showing greater fungistatic activity than did biphenyl. The agar contamination due to spillage was suspected as perhaps being a predominant factor in the marked results obtained with these liquid materials.

In the Petri dish test the isopropyl derivative was the most effective giving complete inhibition of all organisms during the entire

TABLE I

TEST OF VOLATILE FUNGISTATS--PETRI DISH METHOD

Fungistat	Test Organism**	Days of Incubation 3	6	10	13	19	24	28
None	A	1+	4+	4+	4+	4+	4+	4+
	B	+	3+	3+	4+	4+	4+	4+
	C	3+	4+	4+	4+	4+	4+	4+
	D	3+	4+	4+	4+	4+	4+	4+
	E	4+	4+	4+	4+	4+	4+	4+
Biphenyl	A	+	3+	3+	3+	3+	3+	3+
	B	0	3+	3+	4+	4+	4+	4+
	C	+	+	+	2+	2+	2+	2+
	D	2+	3+	3+	4+	4+	4+	4+
	E	+	+	+	+	1+	1+	2+
ASC-4	A	+	4+	4+	4+	4+	4+	4+
	B	+	3+	3+	4+	4+	4+	4+
	C	3+	4+	4+	4+	4+	4+	4+
	D	3+	4+	4+	4+	4+	4+	4+
	E	4+	4+	4+	4+	4+	4+	4+
Methyl*	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0
	E	+	+	+	+	+	+	+
Ethyl*	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0
	E	1+	2+	2+	2+	2+	2+	2+
n-Propyl*	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0
	E	1+	2+	2+	2+	2+	2+	3+
Isopropyl*	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0
	E	0	0	0	0	0	0	0
n-Butyl*	A	0	0	+	+	+	1+	2+
	B	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0
	D	0	0	0	0	0	0	0
	E	+	+	+	+	+	1+	1+

* Dialkyl dichlorosuccinate

**A Diplodia natalensis
B Phomopsis citri
C Penicillium digitatum
D Penicillium italicum
E Rhizopus nigricans

0 No growth
+ Barely perceptible growth
1+ Slight growth
2+ Moderate growth
3+ Heavy growth--little sporulation
4+ Heavy growth--heavy sporulation

test period. The approximate order of effectiveness for all compounds was isopropyl, methyl, ethyl, n-propyl, n-butyl, biphenyl, and ASC-4 from most to least effective, respectively.

Rhizopus nigricans was the most resistant of the five test organisms toward the dichlorosuccinates and the least resistant to the effects of biphenyl. All test organisms grew in the presence of biphenyl with varying degrees of inhibition. In addition to R. nigricans only Diplodia natalensis grew in the presence of the dichlorosuccinates (n-butyl). The dichlorosuccinates, therefore, were effective against a relatively wide range of fungi.

Table II lists the results found by the treated paper technique. In general, the picture was the same for both the Petri dish or treated paper methods. Again the dichlorosuccinates were found to be very effective fungistats showing higher fungistatic activity than biphenyl. The effect of contamination of the agar does not appear to have been an important factor in the results obtained by the Petri dish technique (possible exception was isopropyl derivative).

The methyl and ethyl derivatives gave complete inhibition of all organisms in the second test. The n-propyl derivative completely inhibited all but R. nigricans which grew poorly in its presence. The isopropyl and n-butyl compounds completely inhibited all but R. nigricans and D. natalensis. After 14 days of incubation the treated papers were removed from the jars and the jars reincubated with loosened covers for seven more days. Readings at the end of this period showed little or no

TABLE II

TEST OF VOLATILE FUNGISTATS--FRUIT JAR METHOD

Fungistats	Test Organism**	Days of Incubation 3	7	10	14	21***
None	A	+	4+	4+	4+	4+
	B	+	3+	3+	4+	4+
	C	4+	4+	4+	4+	4+
	D	3+	4+	4+	4+	4+
	E	4+	4+	4+	4+	4+
Biphenyl	A	0	3+	3+	3+	3+
	B	0	2+	2+	2+	3+
	C	0	+	1+	2+	2+
	D	+	3+	3+	3+	3+
	E	0	0	0	0	4+
Methyl*	A	0	0	0	0	0
	B	0	0	0	0	0
	C	0	0	0	0	0
	D	0	0	0	0	0
	E	0	0	0	0	0
Ethyl*	A	0	0	0	0	0
	B	0	0	0	0	0
	C	0	0	0	0	0
	D	0	0	0	0	0
	E	0	0	0	0	0
n-Propyl*	A	0	0	0	0	0
	B	0	0	0	0	0
	C	0	0	0	0	0
	D	0	0	0	0	0
	E	0	+	+	+	+
Isopropyl*	A	0	+	1+	2+	2+
	B	0	0	0	0	0
	C	0	0	0	0	0
	D	0	0	0	0	0
	E	0	0	+	+	+
n-Butyl*	A	0	1+	3+	3+	4+
	B	0	0	0	0	0
	C	0	0	0	0	0
	D	0	0	0	0	0
	E	1+	2+	3+	3+	3+

* Dialkyl dichlorosuccinate

**A Diplodia natalensis
B Phomopsis citri
C Penicillium digitatum
D Penicillium italicum
E Rhizopus nigricans

0 No growth
+ Barely perceptible growth
1+ Slight growth
2+ Moderate growth
3+ Heavy growth--little sporulation
4+ Heavy growth--heavy sporulation

*** Treated sheets removed after 14 days of incubation.

significant recovery of the organisms which had been exposed to the dichlorosuccinates. The same effect was true of the biphenyl treatment with the exception of R. nigricans which had been completely inhibited but grew rapidly after the biphenyl was removed.

The order of effectiveness as related to chemical structure showed that a decrease in toxicity occurred with an increase in the number of carbon atoms in the alkyl group. This is not the expected result in increasing the chain length of an alkyl group on a toxic nucleus and leads one to suspect that the differences in volatility were more important. The position of the isopropyl compound in respect to the other derivatives in this test was about that which would be predicted on the basis of other work on alkyl substitution groups. Usually the n-configuration has proven to be more effective than the iso-configuration of a particular alkyl group.

Figure 1 presents graphically the results of the tests on volatility obtained by exposing a set of treated sheets. It can be seen that the dichlorosuccinates decreased in volatility with an increase in the number of carbon atoms in the alkyl group. Perhaps of greater interest was their position in respect to biphenyl. Two of the alkyl derivatives (n-propyl and n-butyl) showed a lower rate of loss than biphenyl but had greater fungistatic properties. The lower volatility and greater effectiveness of these materials presents a favorable picture in respect to commercial application. It is true the methyl and ethyl derivatives were better fungistats but the more favorable volatility picture would likely offset the slight improvement in fungistatic properties noted, especially in the case of n-propyl compound.

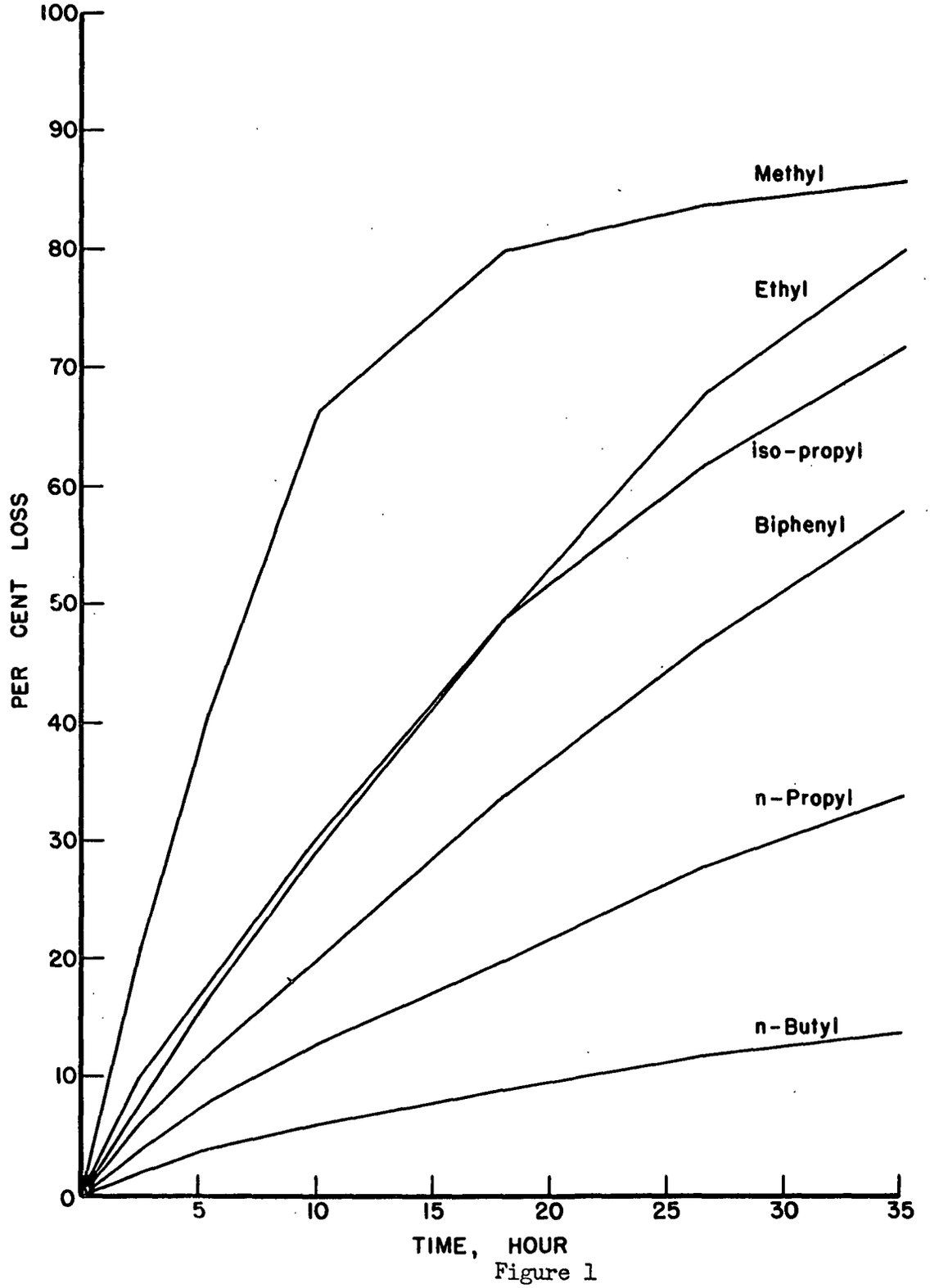

Figure 1

Loss of Fungistat from Exposed Sheets

Also of importance in considering commercial possibilities is the factor of human toxicity. The only toxicological data noted on the dichloro-succinates (1) gave an acute toxicity L_oD_{50} value of 450 mg./kg. for the dimethyl derivative using rats as the test animals. Biphenyl in a similar test had an L_oD_{50} of 3280 mg./kg. An L_oD_{50} of 1000 mg./kg. is considered to be a reasonable figure in roughly dividing the "toxic" materials from the "nontoxic" in this type of test. Therefore, the methyl compound would be questionable as a commercial material from a toxicological standpoint and in turn casts a certain degree of doubt on the other members of the group.

A comparison of the two testing techniques used shows them to be in good agreement in respect to the fungistatic evaluation; however, the newer technique was preferred for a number of reasons. The new method had the advantage of a controlled dosage which could be scaled to actual commercial rates as a base line of comparison. The possibility of spillage and direct contact of the organism and fungistat was eliminated. The equipment needs were simple and inexpensive. Time requirements were about the same for both methods. The measure of volatility was obtained with very little additional time or effort and aided in the evaluation of the materials. The treated paper method, however, would not be applicable to materials which could not be dissolved in volatile solvents.

Several aspects concerning the treatment of the paper sheets should be noted. The variations between individual sheets of a replicate

group was checked using a 30% biphenyl in acetone solution. The mean biphenyl pickup was 83.2 mg. per sheet with a standard deviation of 3.45 mg. The coefficient of variation, therefore, was 4.15% indicating a reasonable degree of uniformity between individual sheets. On the basis of this result the sheets were dipped individually but weighed in groups of ten which decreased the weighing time a great deal. It was found necessary, however, to replace the dipping solution with fresh stock after each 10 sheets. The loss of acetone from the solution increased the concentration of the fungistat to a detectable extent beyond the time interval required to dip 10 sheets. Table III presents the fungistatic content of the sheets used in this study. Since the goal was 85 mg./sheet it can be seen that with four of the six materials very good agreement with this goal was obtained. The ethyl compound was somewhat high; however, only one of the dichlorosuccinate compounds(isopropyl) was used to estimate the concentration of stock which would be needed in the final test and it is surprising they varied so little. The use of isopropyl in the preliminary tests caused a shortage of this compound when the final stocks were made and forced us to use a less concentrated solution of this material. The isopropyl content, therefore, was lower than that of the other four dichlorosuccinates. It is not believed that the lower concentration was the cause of lowered toxicity in the second test of the isopropyl compound, however. In comparing the two test methods, the large reduction in the quantity of fungistat applied between the first and second test (1,000 mg. down to 85 mg.) did not show a reduction in effectiveness with the dichlorosuccinate derivatives. On the basis of this comparison it does not seem likely that the small differences in pickup obtained would affect

the fungistatic results. The precision of treatment appears to be well within the limits imposed by variability of the test fungi.

TABLE III

FUNGISTAT CONTENT OF TREATED SHEETS

Fungistat	Weight in mg. Fungus Test Sheets	Exposed Sheets
Biphenyl	85.4	83.6
Methyl*	81.1	79.2
Ethyl*	94.2	91.8
n-Propyl*	82.5	83.3
Isopropyl*	70.5	68.5
n-Butyl*	84.6	81.7

(Each figure is average of ten sheets.)

* Dialkyl dichlorosuccinate

CONCLUSIONS

1. The dialkyl dichlorosuccinate compounds were found to be more effective fungistats than biphenyl against five species of fungi which cause deterioration of fruits during storage. 2 Two of the dichlorosuccinates, n-propyl and n-butyl, were found to dissipate from exposed sheets at a slower rate than biphenyl which would be a favorable property in commercial application. However, indications are that the toxicological aspects of the dichlorosuccinates versus biphenyl may not be so favorable from the

because of evidence of dimethyl —

standpoint of commercial usage. The testing method using fruit jars and sheets treated with the fungistatic material was favored over the older Petri plate-glass cup method.

LITERATURE CITED

1. Food Engineering 26, no. 10:93-94, 155 (Oct., 1954).

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